Non-Invasive Method for Quantitative Evaluation of Exogenous Compound Deposition on Skin

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Topical application of active compounds on skin is common to both pharmaceutical and cosmetic industries. Quantification of the concentration of a compound deposited on the skin is important in determining the optimum formulation to deliver the pharmaceutical or cosmetic benefit. The most commonly used techniques to date are either invasive or not easily reproducible. In this study, we have developed a noninvasive alternative to these techniques based on spectrofluorimetry. A mathematical model based on diffusion approximation theory is utilized to correct fluorescence measurements for the attenuation caused by endogenous skin chromophore absorption. The limitation is that the compound of interest has to be either fluorescent itself or fluorescently labeled. We used the method to detect topically applied salicylic acid. Based on the mathematical model a calibration curve was constructed that is independent of endogenous chromophore concentration. We utilized the method to localize salicylic acid in epidermis and to follow its dynamics over a period of 3 d. ***Key words: fluorescence spectroscopy/salicylic acid/optical modelling. J Invest Dermatol 118:295–302, 2002***

Simple, fast, and noninvasive *in vivo* methods for estimating exogenous compound deposition on skin are invaluable tools in understanding the pharmacokinetics of topical formulations. In order to quantify the deposition of a substance externally applied on skin several techniques have been developed. Of those the most commonly used to date are invasive (biopsy, microdialysis) or minimally invasive (Benfeldt, 1999). Tape stripping is a minimally invasive method based on the removal of stratum corneum layers by successive tape applications and removals and the subsequent analysis of the removed material on the tape strips for drug content. Tape stripping followed by solvent extraction and high performance liquid chromatography (HPLC) has been used by many investigators (Pirola *et al*, 1998; Schwarb *et al*, 1999; Tsai *et al*, 1999). Another variation of this method is to use a radiolabeled equivalent of the compound to be deposited and measure the radiation on the tape strips by liquid scintillation methods (Bommannan *et al*, 1992; Wester *et al*, 1998). Such radiolabeling methods have been reported to be expensive and time consuming (Schaefer and Redelmeier, 1996). Tape stripping methods appear to have many limitations, such as the inability to provide serial sampling from the same site. Furthermore, the quantity of corneocytes removed is not linearly proportional to the number of tape strips (Weigmann *et al*, 1999). Several factors can influence the quantity of stratum corneum removed by each piece of tape, e.g., the removing force and manner, the hydration of skin, anatomical site, and interindividual variation (Marttin *et al*, 1996). Finally, the removal of corneocyte layers can be incomplete due to the presence of microscopic groove structures (van der Molen *et al*, 1997) also known as microrelief.

In vivo fluorescence spectroscopy has been proposed as a noninvasive alternative for the assessment of deposition or penetration of substances into the skin (Sennhenn *et al*, 1993; Rhodes and Diffey, 1997; Wagnieres *et al*, 1998). These reports have not taken into consideration phenomena of photon reabsorption and scattering in the tissue, however, and therefore such measurements may vary a lot between people of different skin types and between different anatomical sites of the same individual. Absorption and scattering corrections of fluorescence signals have been developed for studies of native skin fluorescence (Wu *et al*, 1993; Zeng *et al*, 1995).

Salicylic acid is widely used in topical preparations to remove excess scales in the treatment of hyperkeratotic conditions, such as psoriasis and seborrheic dermatitis. When added to cosmetic formulations, it can increase cell renewal and boost the antimicrobial capabilities of the formulation. Due to its keratolytic properties salicylic acid is present in a wide range of wart and callus remedies as well as in antidandruff shampoos. Its fluorescence properties allow for its detection as an impurity in solid formulations of acetyl-salicylic acid (Schenk *et al*, 1972; Street and Schenk, 1981). Neubert *et al* (1990) have used the tape stripping technique combined with fluorescence spectroscopy of the extracted solution to measure salicylic acid deposition on skin.

The goal of this investigation was to evaluate the feasibility of quantifying noninvasively *in vivo* the concentration of topical deposition of a model fluorescing compound (salicylic acid) on human skin independent of anatomical location and interindividual differences in skin color. To this end we developed a method based on fluorescence spectroscopy with corrections for photon reabsorption and scattering by the skin tissue.

MATERIALS AND METHODS

Volunteers and application method In all experiments 10 μl of each solution were applied evenly on predetermined circular areas (2.9 cm diameter) on the volar forearm of each volunteer.

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Abbreviation: PMT, photomultiplier.

In the first set of experiments a series of dilutions of an aqueous formulation containing salicylic acid (3% wt/vol initial concentration) were applied to the forearms of 10 healthy volunteers (ages 20–50 y). The surface concentrations of salicylic acid applied ranged between 1 and 25 μg per cm^2. Measurements were acquired from treated skin sites after solvent evaporation, as well as from adjacently located untreated and placebo-treated sites. All sites of measurement were free of skin diseases, uneven skin tones, sunburn, tattoos, and scars.

In another set of experiments a salicylic acid solution (initial surface concentration of salicylic acid was 3 μg per cm^2) was applied on both volar forearms of two volunteers (a total of four sites). Square pieces (2.5 cm × 2.5 cm) of adhesive tape (#5413, 3M, St. Paul, MN) were applied gently on the treated sites. The tapes were stripped after 1 min. A total of 20 tapes were applied sequentially. Fluorescence measurements were acquired before and after salicylic acid application as well as after each tape strip.

In a third set of experiments we followed salicylic-acid-induced fluorescence over time. Two solutions (10 μl each) of different salicylic acid concentrations (3 μg per cm^2 and 10 μg per cm^2) were applied on both volar forearms of 10 volunteers (a total of 20 sites per treatment). Fluorescence measurements were acquired before and after application of salicylic acid, as well as once a day from then on until the fluorescence signal reduced to levels of native skin fluorescence.

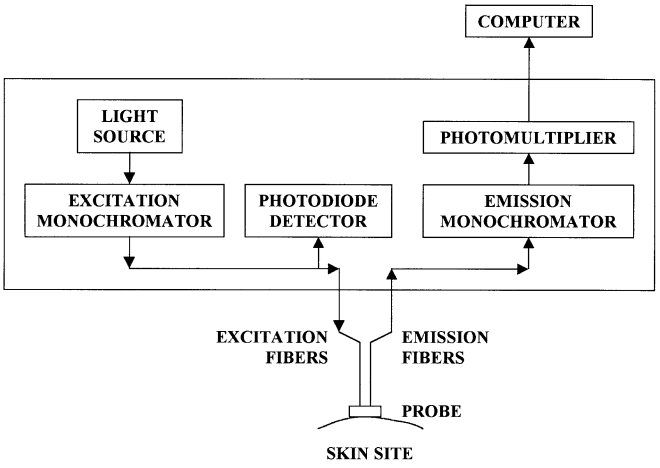

Figure 1. Schematic diagram of the experimental setup.

In a final set of experiments five different dilutions of salicylic acid solution (corresponding to surface concentrations ranging between 0.5 and 5 μg per cm^2) were applied on the volar forearms of nine volunteers. Fluorescence measurements were acquired before and after application of salicylic acid. The sites were tape stripped gently once, as described above. The tapes were placed into a 15 ml Falcon polypropylene tube (Becton Dickinson Labware, Franklin Lakes, NJ). Methanol (3 ml in each tube, Burdick & Jackson, Muskegon, MI) was used to extract the salicylic acid from the tapes. The solutions were sonicated for 30 min and stored in a refrigerator at $< 5°C$ before HPLC analysis for quantification.

Analytical methods The HPLC system was equipped with an ultraviolet–visible detector and a photodiode array detector, capable of monitoring absorption at 300 nm, a column oven, and an automatic sampling system (HP 1100 HPLC). The HPLC column used was a reverse phase Selectosil C18 column (150 × 3.2 mm internal diameter, 5 μm average particle size; from Phenomenex). The mobile phase consisted of 70% tetramethylammonium hydroxide solution, 15% acetonitrile, and 15% methanol. Glacial acetic acid (1 ml) was added to the mobile phase to improve the separation resolution. The flow rate was 0.4 ml per min and the column was kept at 40°C. The absorption was measured at 300 nm. Calibration was done by running standards in every sample set by linear regression of peak area *versus* concentration (standard concentrations 0, 0.5, 1, 5 μg per ml salicylic acid in methanol). The mean retention time for salicylic acid was 2.8 min and free from any potential biologic interference. A typical run took less than 7 min. The sample extract was filtered through a 0.45 mm PTFE filter (Whatman), discarding the first 1 ml into an autosampler vial. The duplicates typically agreed within 3% of their average following this method.

In vivo fluorescence spectroscopy was performed using a SPEX SkinSkan spectrofluorimeter (SPEX Industries, Edison, NJ). Excitation radiation from a 125 W xenon arc lamp was filtered through an excitation double monochromator (200–660 nm wavelength range and 1200 grooves per mm grating) and was focused onto one leg of a bifurcated quartz fiber optic bundle (**Fig 1**). The fiber optic bundle (2 mm total diameter) consisted of 62 fibers (214 μm in diameter each) and was used to deliver excitation radiation to the skin and emission radiation from the skin back to the spectrofluorimeter. The excitation and emission fibers were randomly dispersed. Measurements were acquired by placing the fiber optic probe in contact with the skin sample. In a subset of experiments a flat quartz spacer (1.2 mm thick) was placed between the fibers and the skin. In all cases collected radiation was filtered through an emission double monochromator (200–680 nm wavelength range and 1200 grooves per mm grating) and was quantified by a photomultiplier (PMT) detector (R1527, Hamamatsu, Hamamatsu City, Japan). The xenon arc lamp output was measured as reference at excitation by an ultraviolet (UV) enhanced silicon photo-

Table I. The fluorescence maxima of (i) salicylic acid powder, (ii) skin treated with a formulation including 3% salicylic acid (formulation A), (iii) skin treated with the same formulation without salicylic acid (formulation B; placebo), and (iv) untreated skin, as observed at the corresponding fluorescence spectra[a]

	Designation	Excitation (nm)	Emission (nm)	Relative intensity (a.u.)
Powder	a'	270	370	0.14
	b′	280	430	0.17
	c′	350	370	0.28
	d′	350	430	0.18
Formulation A on skin	a	290	400	0.82
(salicylic acid)	b	290	420	1.00
	c	350	400	0.56
	d	350	420	0.86
Formulation B on skin	e	270	310	0.02
(placebo)	f	310	370	0.13
	g	360	430	0.21
	h	370	460	0.17
Untreated skin	i	300	340	0.06
	j	340	390	0.04
	k	360	430	0.05
	l	370	460	0.06

[a]The fluorescence intensities have been normalized to the maximum one (designated b).

diode detector. The spectrofluorimeter was controlled by a computer using DataMax spectroscopy software based on Grams/32® (Galactic Industries, Salem, NH). The user was able to control excitation and emission wavelength for single point measurements or scans, as well as the high voltage bias of the PMT. Before each set of measurements the instrument was spectrally calibrated for excitation and emission following the manufacturer's instructions. Briefly, for excitation calibration a reference scan of the light source was acquired and the absolute maximum was set to 467 nm (characteristic of the xenon arc source). For emission calibration a synchronous scan was acquired of a white diffuse reflectance calibration plate (Minolta CR-200/CR-300) and the absolute maximum of the signal was set to 467 nm. The Minolta plate has optical properties similar to a barium sulfate surface, but was preferred over the latter due to its structural integrity. The chromatic resolution of the spectrofluorimeter was ±1 nm.

Wavelength optimization The optimum excitation–emission pair for measurements of fluorescent compound deposition on skin should be at a fluorescence maximum with minimal background interference and minimal self-absorption. Interfering signals may arise from either skin native fluorescence (Leffell *et al*, 1988; Zeng *et al*, 1995; Kollias *et al*, 1998; Brancaleon *et al*, 1999) or fluorescence of formulation components other than salicylic acid. In order to identify the optimum excitation–emission pair, we acquired the fluorescence spectra of the following: (i) salicylic acid powder; (ii) skin treated with a formulation including 3% salicylic acid (formulation A); (iii) skin treated with the same formulation without salicylic acid (formulation B; placebo); and (iv) untreated skin. The spectra were acquired as a series of 31 emission scans (260–560 nm) with excitations ranging between 240 nm and 550 nm. Measurements were recorded every 10 nm. **Table I** summarizes all local maxima of the corresponding spectra. The spectrum obtained from salicylic acid powder is very similar to the one obtained from formulation A with the maxima only slightly shifted and the relative intensities changed. The fluorescence maxima of salicylic acid in formulation A are 3–42 times stronger than the signals from other ingredients in the formulation (see maxima of formulation B). Two of the salicylic acid fluorescence maxima (designated c and d) are overlapping with broad skin autofluorescence maxima (j, k, and l). There is minimal skin autofluorescence at the excitation–emission wavelength pairs of the other two maxima of salicylic acid (a and b). Finally, of these two we can obtain a better dynamic range if we use the strongest one (designated b).

Salicylic acid powder absorbance was measured in the UV. A single narrow band was detected centered at 314 nm with a bandwidth at half maximum of 16 nm. Absorbance was insignificant at 290 nm or 420 nm (<0.1 absorbance units). We concluded that the wavelength pair of excitation at 290 nm and emission at 420 nm was the ideal choice for measuring salicylic acid fluorescence, as it resulted in a strong signal with minimal interference from background (for salicylic acid concentrations > 0.1 μg per cm^2) and insignificant self-absorption phenomena. **Figure 2** shows the difference in excitation spectra before and after treatment of volar forearm skin with 10 μg per cm^2 salicylic acid.

Optical modeling The following model has been developed to correct the recorded fluorescence signal for variations due to local differences in the optical properties of the skin, namely absorption and scattering. For simplicity the model is based on a first-order approximation of these optical phenomena. We assume that the compound to be quantified has been deposited in the first few micrometers (<10 μm) of the stratum corneum and is bound to it (**Fig 3**). With the excitation monochromator set at λ_{exc}, radiation is delivered to the skin surface by the excitation fibers. Due to the mismatch between the index of refraction of air (n_{air} = 1) and that of the stratum corneum ($n_{SC} \approx 1.5$) (van Gemert *et al*, 1989), a fraction of the incident radiation will be returned back as specular

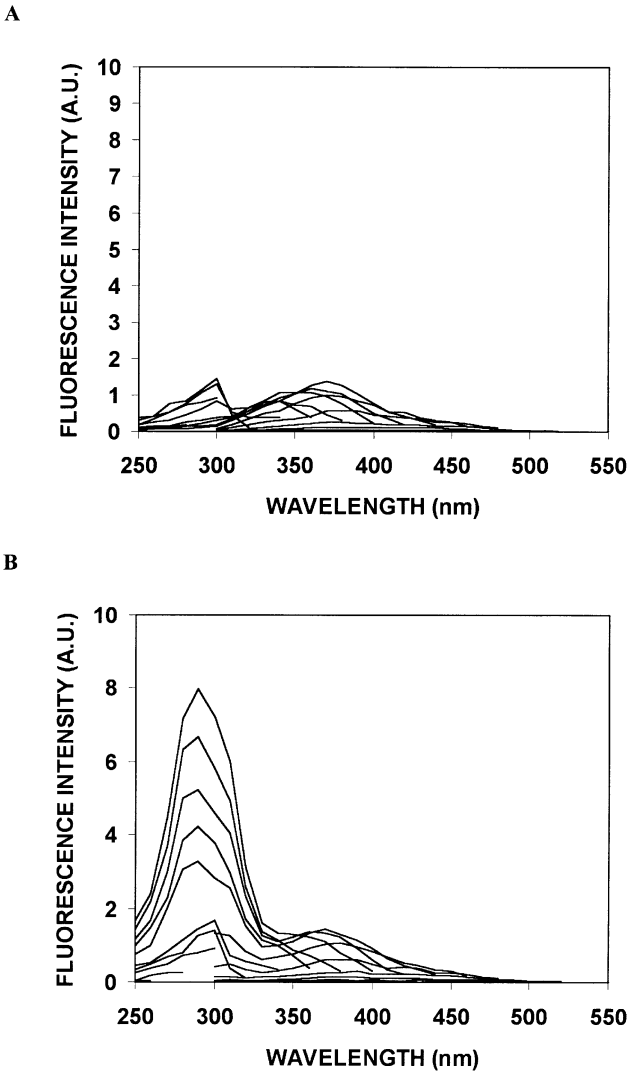

Figure 2. A series of 31 excitation scans on volar forearm skin before and after treatment with 10 μg per cm^2 salicylic acid. (*A*) Before treatment; (*B*) after treatment. The emission wavelength was increased for every successive scan by 10 nm starting at 260 nm and finishing at 560 nm. The two plots are shown in the same scale to demonstrate the increase of the fluorescence signal at 290 nm excitation. Using this excitation and measuring at 420 nm emission the interference from skin native fluorescence is minimal.

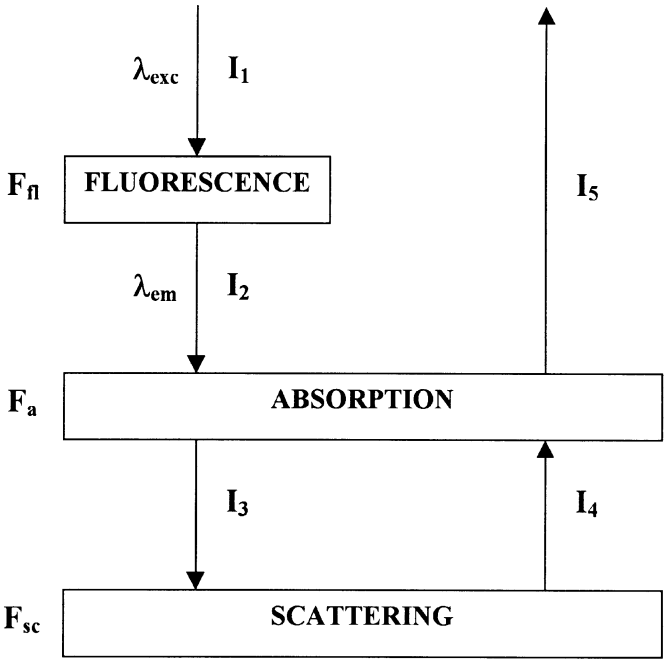

Figure 3. Schematic model of the optical phenomena occurring when excitation radiation is delivered to skin with a superficially deposited fluorescent compound.

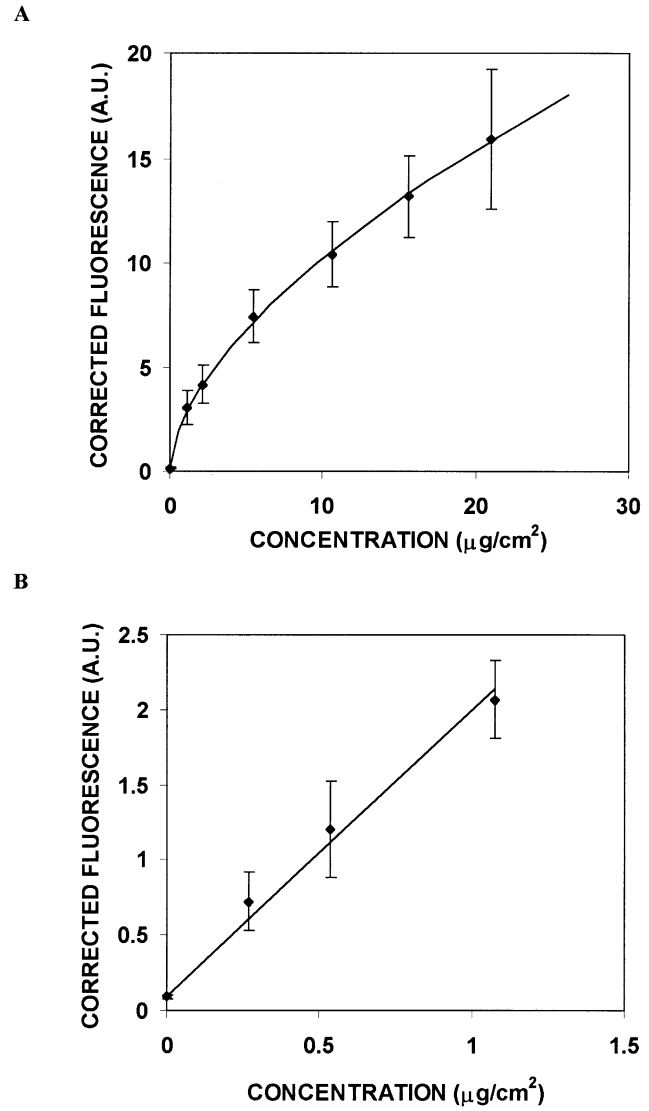

Figure 4. The relation between corrected fluorescence measurements and surface concentration of salicylic acid for moderate and low concentrations. (*A*) Moderate concentration; (*B*) low concentration. Data show average values of measurements acquired from 10 volunteers with skin types ranging from I to IV. Error bars indicate standard deviations. The solid lines represent the fitted power ($C = 0.21 \times (F - 0.09)^{1.68}$) and linear ($C = 0.53 \times (F - 0.09)$) curves for moderate and for low concentrations, respectively. Note that the corrected fluorescence value at zero concentration is not zero (0.09 a.u.). This value corresponds to native skin fluorescence.

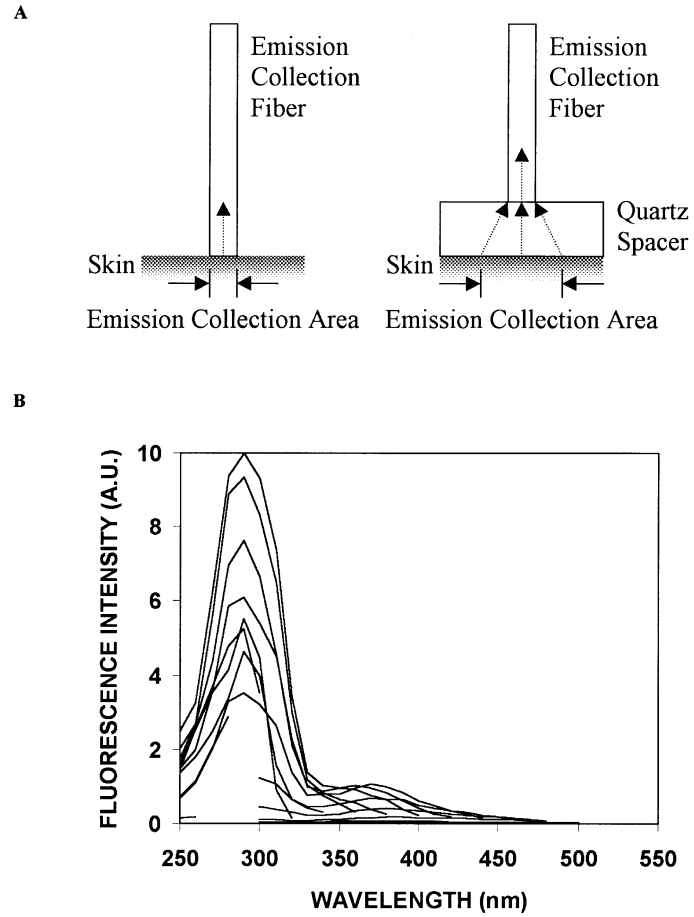

Figure 5. Excitation spectra using a quartz spacer. (*A*) The emission collection angle is being enhanced using a quartz spacer between the probe and the skin, resulting in a larger emission collection area. (*B*) A series of 31 excitation scans on volar forearm skin treated with 10 μg per cm^2 salicylic acid with a quartz spacer placed between the probe and the skin. The emission wavelength was increased for every successive scan by 10 nm starting at 260 nm and finishing at 560 nm.

reflection (Hecht and Zajac, 1979). This fraction can be calculated from the Fresnel equation for normal incidence of light on the interface, $f = [(n_{air}n_{SC})/(n_{air} + n_{SC})]^2$ (Jenkins and White, 1976; Hecht and Zajac, 1979), and is about 4%. The radiation that passes through the interface (with intensity I_1) is absorbed by the molecules of the fluorescing compound and causes them to emit radiation at longer wavelengths (λ_{em}). The fluorescence is emitted uniformly in all directions. The intensity of the emitted radiation in the forward direction (towards the dermis) is $I_2 = I_1F_{fl}$, where F_{fl} is a proportionality factor depending on the concentration of the fluorophore, the quantum yield at λ_{exc}, the extinction coefficient at λ_{exc}, and the geometry of the probe. When λ_{exc} = 290 nm the excitation radiation that is not attenuated by the exogenous compound is absorbed by the proteins in the stratum corneum, e.g., the amino acids tryptophan and tyrosine absorb strongly in the range 280–290 nm (Anglin *et al*, 1972; Anderson and Parrish, 1981). The forward part of the emitted radiation travels through the epidermis, where it is partially absorbed by epidermal chromophores, mainly by melanin (Anderson and Parrish, 1981; Kollias and Baqer, 1985; 1987). When it reaches the basal layer a fraction of it gets reflected back at the interface and the rest enters the dermis (with intensity $I_3 = I_2F_a$, where F_a is a proportionality factor depending on the concentration of the absorber and its extinction coefficient at λ_{em}). In the dermis collagen scatters the entering radiation, whereas hemoglobin attenuates its intensity (Anderson and Parrish, 1981; Andersen, 1997) (in the case of λ_{em} = 420 nm hemoglobin attenuation is strong). A significant fraction (F_{sc}) scatters back to the epidermis ($I_4 = I_3F_{sc}$), where it is absorbed again partially by melanin (by a factor of $1 - F_a$) and finally reaches the surface of the skin ($I_5 = I_4F_a$), where it is collected by the emission fibers and measured by the instrument with the emission monochromator set at λ_{em}. The fiber bundle has a narrow collection angle (± 10°) and therefore only radiation scattered within this angle could be measured. Combining the equations together we get

$$I_5 = F_aI_4 = F_{sc}F_aI_3 = F_{sc}F_a^2I_2 = F_{fl}F_{sc}F_a^2I_1 \quad (1)$$

By setting the excitation and emission monochromators at the same wavelength ($\lambda_{exc} = \lambda_{em}$) we measure the diffuse reflectance of the skin at this wavelength. Assuming that the extinction coefficient of the absorbing chromophore is negligible at this wavelength ($I_2' = I_1$) then

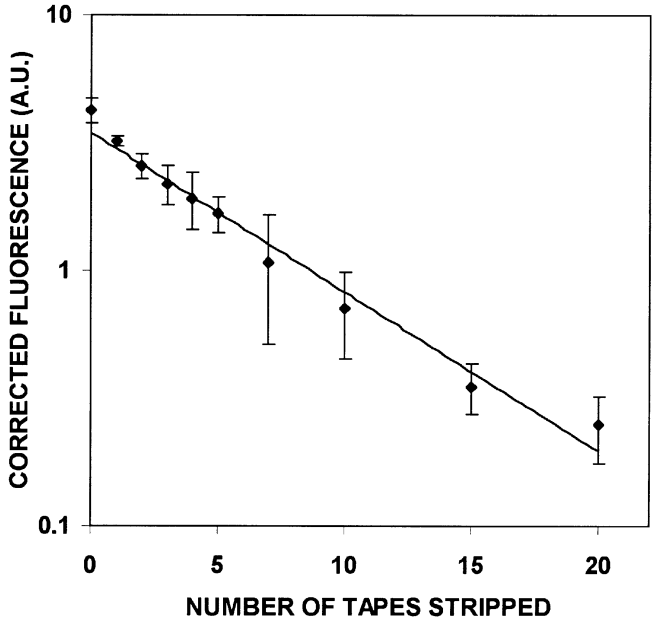

Figure 6. Fluorescence measurements of salicylic acid on skin following tape stripping. The data show average values of measurements from four sites on two volunteers. Error bars indicate the standard error of the mean. The fitted line is given by $F = 3.57 \times \exp(-0.14n)$.

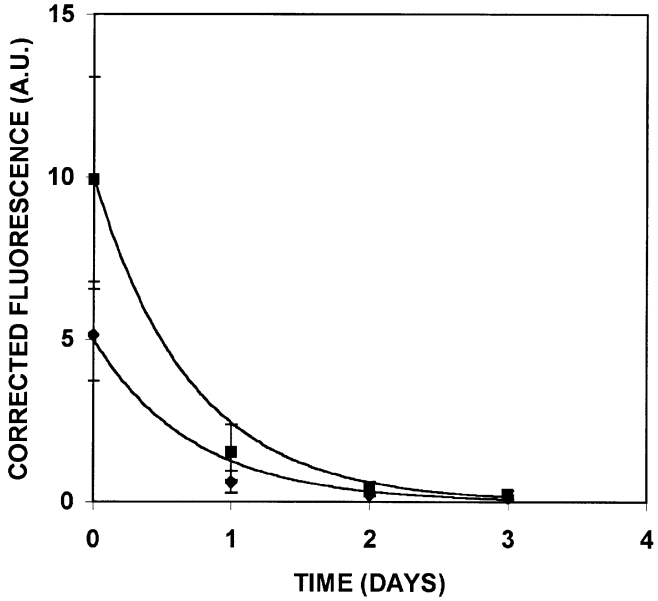

Figure 7. Salicylic acid concentration on skin decreases exponentially over time as evidenced by the decrease in fluorescence. The squares represent average corrected fluorescence intensity values for an initial surface concentration of 10 μg per cm^2 and the diamonds the corresponding values for an initial surface concentration of 3 μg per cm^2. Error bars indicate the standard error of the mean. The solid lines represent the fitted exponential curves given by $F = 9.9 \times \exp(-1.4t)$ and $F = 5.1 \times \exp(-1.4t)$, respectively.

$$I'_5 = F_a I'_4 = F_{sc} F_a I'_3 = F_{sc} F_a^2 I'_2 = F_{sc} F_a^2 I_1 \quad (2)$$

From (1) and (2)

$$\frac{I_5}{I'_5} = F_{fl} \quad (3)$$

As we mentioned, F_{fl} depends only on the concentration of the fluorophore and its quantum yield at λ_{exc}, and therefore the ratio is independent of tissue absorption and scattering, as well as fluctuations of excitation radiation intensity. For low concentrations the assumptions of the Beer–Lambert law are well approximated and the above ratio is linearly proportional to the fluorophore concentration.

Therefore, by normalizing the fluorescence signal by the diffuse reflectance at the emission wavelength we can correct for absorption and scattering phenomena by native skin constituents. After placing the probe on the site of interest, the intensity of the fluorescence emission (at λ_{ex} = 290 nm and λ_{em} = 420 nm) was recorded, with the high voltage of the PMT detector kept at 950 V. Then the diffuse reflectance value was recorded at 420 nm at the same site without moving the probe. The PMT high voltage was reduced to 400 V to avoid detector saturation.

RESULTS

The corrected fluorescence intensities in arbitrary units (a.u.) measured for different surface concentrations of salicylic acid are shown in **Fig 4**. In general the calibration curve can be fitted by a power law (Rhodes and Diffey, 1997):

$$C = a(F - F_0)^b \quad (4)$$

where C is the surface concentration of salicylic acid in μg per cm^2, F is the corrected fluorescence intensity at the selected excitation and emission wavelengths in a.u., F_0 is the corrected value of native skin fluorescence, and a and b are parameters to be fitted. For the case of salicylic acid and for excitation 290 nm and emission 420 nm we found $F_0 = 0.09$ a.u., $a = 0.21$ μg per cm^2 per a.u., and $b = 1.68$. The detection limit was 0.1 μg per cm^2. The saturation phenomenon determines the upper limit for measurements to be about 20 μg per cm^2. The PMT provides a dynamic range that is greater than the apparent saturation of the signal at high concentrations. Therefore, this apparent saturation most probably reflects a chemical saturation of the stratum corneum with salicylic acid. For concentrations below 1 μg per cm^2 the relation between the concentration and the fluorescence can be assumed to be linear (i.e., we approach the limit of the Beer–Lambert law):

$$C = a'(F - F_0) \quad (5)$$

where $a' = 0.53$ μg per cm^2 per a.u. The average coefficient of variation of the fluorescence intensity before the correction was 67%. After the correction it was reduced to 19%. The calibration curves of **Fig 4** were validated with samples of known concentration. The error was calculated to be less than 10% for both low concentrations and moderate concentrations.

We tested the assumption that salicylic acid is localized mostly in the top layers of the epidermis first by placing a 1.2 mm thick quartz flat plate between the fiber probe and the treated skin sites, thus enhancing the collection angle of the fiber bundle (**Fig 5*A***), and second by applying the tape stripping method. **Figure 5(*B*)** shows a series of excitation spectra similar to those of **Fig 2** for a skin site treated with 10 μg per cm^2 salicylic acid only this time a quartz spacer was placed between the probe and the skin. Using this configuration we were able to detect fluorescence originating mostly from the superficial layer of the stratum corneum (**Fig 5*A***). The maximum fluorescence intensity was 25% higher than the corresponding measurement without the spacer (**Fig 2**). This provided the first indication that most of the salicylic acid has been deposited on the superficial layer.

Tape stripping of the treated site and measurements of fluorescence between strips provided more evidence that most of the fluorescent source was localized in the first layers of the stratum corneum. It was found that the fluorescence signal decreased exponentially with the number of tapes (**Fig 6**). The data points can be fitted with the equation

$$F = 3.47 \times \exp(-0.14n) \quad (6)$$

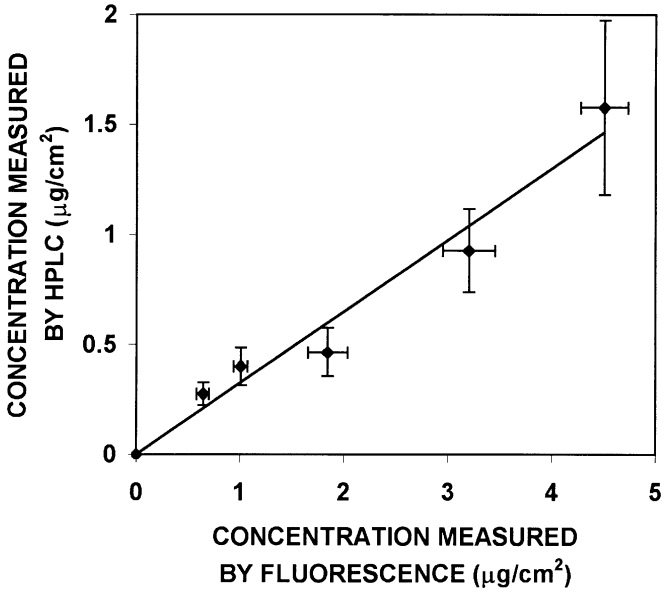

Figure 8. Comparison of the fluorescence method of quantifying salicylic acid on skin with the traditional method of tape stripping followed by HPLC. The data show average values from samples from nine volunteers. Error bars indicate the standard error of the mean. The fitted line is given by $C_{HPLC} = 0.325 \times C_{Fluorescence}$ with $R^2 = 0.967$.

where n is the number of tapes used. Measurements conducted on untreated sites before and after tape stripping showed that native fluorescence with the excitation–emission pair used (290 nm excitation and 420 nm emission) did not change with tape stripping. After 10 tape strips the residual salicylic-acid-induced fluorescence was < 1% of that before.

The salicylic-acid-induced fluorescence was followed daily for two different concentrations until the fluorescence intensity was reduced to < 5% of the initial signal (**Fig 7**). The fluorescence intensity decreased exponentially for both initial surface concentrations over time with the same rate constant, $k = -1.4\ d^{-1}$. Assuming that corneocytes are continuously shed at a constant rate, the exponential decrease is yet another indication of the exponential distribution profile of salicylic acid in the stratum corneum layers. Furthermore, these results indicate that salicylic acid may be used as a marker for exfoliation measurements.

We compared the presented fluorescence method with the commonly used technique of tape stripping followed by extraction and HPLC analysis. The results for five different concentrations of salicylic acid are shown in **Fig 8**. The two methods correlated well ($R^2 = 0.967$); however, for the same salicylic acid concentrations the HPLC method resulted in significantly lower values than the fluorescence measurements. Furthermore, as evidenced by the error bars, towards the higher concentrations the HPLC measurements were more variable than the corresponding fluorescence results.

DISCUSSION

In this study, we present a noninvasive spectrofluorimetric method for quantification of an exogenous fluorophore deposition on skin. The method would be applicable for the quantification of any fluorophore or fluorescently labeled compound applied topically onto the skin. Spectrofluorimetric measurements present no limitations due to molecular size or lipophilicity, as does microdialysis (Benfeldt, 1999). Changes in the molecular environment of the fluorophore, however, can induce changes in its quantum efficiency.

Salicylic acid is ideally suited for quantification on skin because it excites at a wavelength that is not effective in inducing a strong native skin fluorescence. Second, the excitation radiation is strongly attenuated by amino acids (tryptophan absorption maximum at 290 nm; Anglin *et al*, 1972; Anderson and Parrish, 1981), melanin (absorption increasing to shorter wavelengths in UV; Anderson and Parrish, 1981), and urocanic acid (absorption maximum at 280 nm; Hanson and Simon, 1998) and therefore does not penetrate beyond the first few cell layers of the viable epidermis. Third, the extinction coefficient of salicylic acid is relatively small at both the excitation and the emission wavelengths and therefore correction for inner filter effects is not necessary. Finally, the only care that needs to be taken is to ensure that the blood flow through the measured sites remains constant during measurement because of the potentially strong attenuation at the emission wavelength by hemoglobin.

Sweat and sebum are not expected to quench or interfere in any way with the fluorescence of salicylic acid. The fluorescence of salicylic acid was strong whether it was dissolved in aqueous solutions or in a lipid mixture that resembled the composition of sebum (data not shown).

The fluorescence maxima of salicylic acid deposited on skin reported in this study are close to the maxima of the compound in solution. Miles and Schenk (1970) reported that, depending on the solvent, salicylic acid has a fluorescence maximum ranging between 280 nm and 300 nm excitation and around 450 nm emission, consistent with the wavelengths chosen for this study (290 nm excitation and 420 nm emission).

When measuring fluorescence *in vivo*, the signal may be compromised due to radiation absorption by chromophores native to skin. Melanin in the epidermis and hemoglobin in the dermis are the primary chromophores that absorb UV and visible radiation. Their concentration varies between individuals and between different skin sites. Radiation absorption may occur at the excitation and/or the emission wavelengths. This is an important factor to be considered for intrasubject and intersubject consistency of the fluorescence measurements. We have presented a simple algorithm to account for differences in tissue absorption especially at the emission wavelength. In the case of superficially deposited compounds and for the probe geometry we described, we showed that correction for absorption by endogenous chromophores at the emission wavelength is most important. To this end we normalized the measured fluorescence intensity by the diffuse reflectance of radiation at the emission wavelength. We demonstrated experimentally that the ratio of fluorescence to diffuse reflectance is independent of the concentration of absorbing chromophores for Fitzpatrick skin types I–III, as the average coefficient of variation for measurements between subjects was significantly decreased after the correction (from 67% before correction to 19% after correction).

Investigators studying native skin fluorescence have proposed normalization of the fluorescence signal using diffuse reflectance (Wu *et al*, 1993; Zeng *et al*, 1995). This suggestion has been based on theoretical analysis of photon migration using diffusion approximation theory and Monte Carlo simulations. In this study we reached the same conclusion for the measurement of fluorescence signals from compounds applied externally to skin. Another method that accounts for phenomena such as photon reabsorption and scattering is using partial least squares analysis to develop an empirical linear model for fluorescence from a turbid medium such as skin (Durkin and Richards-Kortum, 1996). This method requires a substantial training set of measurements from a tissue phantom with optical properties similar to skin, however.

Using the corrected fluorescence, we demonstrate that for low concentrations of salicylic acid (up to 1 μg per cm^2) the fluorescence signal varies linearly with concentration, whereas for moderate concentrations its fluorescence can be modeled as a simple exponential (for concentrations up to 20 μg per cm^2). The detection limit of this method (0.1 μg per cm^2) was better than that reported for tape stripping followed by extraction and HPLC analysis (1 μg per cm^2) (Pirola *et al*, 1998). Comparison of the two techniques (**Fig 8**) showed that the chromatographic method underestimates the salicylic acid concentrations. This can be

explained by the incomplete removal of all the salicylic acid from the stratum corneum by tape stripping and liquid extraction. Furthermore, the greater variability in the HPLC results possibly arises from the increased probability of experimental error due to the many steps of the procedure (tape stripping, extraction, HPLC analysis) compared to the single step of the fluorescence method.

We demonstrated that most of the salicylic acid is deposited on the first few layers of the stratum corneum. Hence, our method may be useful for evaluating skin desquamation rates, keeping in mind that salicylic acid itself is a keratolytic agent at concentrations higher than 2%, which corresponds to >10 μg per cm^2 surface concentration (Huber and Christophers, 1977; Roberts *et al*, 1980; Loden *et al*, 1995; Kligman and Kligman, 1998). The currently used methods to evaluate desquamation rates include cohesography (Marks *et al*, 1977), surfometry (Marks *et al*, 1977), disappearance of dansyl chloride fluorescence staining (Roberts and Marks, 1980), silver nitrate staining (Roberts and Marks, 1980; Roberts *et al*, 1980), and quantification of the stratum corneum material removed by tape stripping (Loden *et al*, 1995). The use of salicylic acid fluorescence to this end provides a nondestructive, noninvasive, and nontoxic alternative.

Our observation that after topical application the salicylic acid concentration decreases exponentially with skin depth is in agreement with microdialysis studies (Benfeldt and Serup, 1999). Similar concentration profiles have been reported before for other molecules and have been attributed to the larger spaces in the upper laminae of the stratum corneum rather than to small diffusion coefficients of the deposited molecules (Tregear, 1966). The stratum corneum has been reported to act as a reservoir for salicylic acid delivery (Rougier *et al*, 1985). Penetration of salicylic acid is measurable only when the stratum corneum is perturbed by tape stripping or delipidation (Birmingham *et al*, 1979; Benfeldt, 1999). Otherwise, most of the deposited compound remains bound on the stratum corneum (Neubert *et al*, 1990; Bommannan *et al*, 1992; Loden *et al*, 1995; Wester *et al*, 1998) until it gets removed due to natural skin turnover. This fact was demonstrated by the exponential decrease of fluorescence over a period of a few days. The lag time for measurable salicylic acid penetration in full thickness human skin is about 20 h and for epidermis alone about 9 h at pH 4 (Harada *et al*, 1993), times well above the time it takes for the fluorescence measurement.

Although in our experiments we have used a compound that is deposited on the stratum corneum, the spectrofluorimetric method presented here can also be applied for the quantitation of compounds that penetrate the epidermal layers. In the latter case, however, it is more difficult to get information about the localization of the fluorescent compound in the skin *in vivo* and noninvasively. Measurement of the penetration of the excitation wavelength may provide some information on the localization of the compound. The time dependence of the fluorescence signal may also give information about the penetration of the compound through skin. If the wavelengths are suitable *in vivo* fluorescence confocal microscopy (Bussau *et al*, 1998) may be useful. Otherwise one has to rely on more invasive methods, namely histologic evaluations.

In conclusion, we have demonstrated that fluorescence spectroscopy can be used to quantify the accumulation of a fluorescent compound on a biologic tissue *in vivo*. The limitation is that interference from native skin fluorescence should be minimal at the wavelengths of interest. As an example we measured salicylic acid deposited on skin. The major advantages of this method are: (i) it is sensitive (low detection limit); (ii) it is noninvasive; (iii) it shows good reproducibility; and (iv) it is fast and simple to implement compared to the methods available to date.

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